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Identification and Characterization of CdgB, a Diguanylate Cyclase Involved in Developmental Processes in *Streptomyces coelicolor*▽

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We describe the identification and functional characterization of *cdgB* (SCO4281), a recently discovered target of BldD, a key regulator of morphological differentiation and antibiotic production in the mycelial bacteria of the genus *Streptomyces*. *cdgB* (cyclic dimeric GMP [c-di-GMP] B) encodes a GGDEF-containing protein that has diguanylate cyclase activity *in vitro*. Consistent with this enzymatic activity, heterologous expression of *cdgB* in *Escherichia coli* resulted in increased production of extracellular matrix in colonies and enhanced surface attachment of cells in standing liquid cultures. In *Streptomyces coelicolor*, both overexpression and deletion of *cdgB* inhibited aerial-mycelium formation, and overexpression also inhibited production of the antibiotic actinorhodin, implicating c-di-GMP in the regulation of developmental processes in *Streptomyces*.

Bis-(3′-5′)-cyclic dimeric GMP (c-di-GMP) was discovered in 1987 (33). However, its importance as a second messenger influencing the choice between motile and sessile "lifestyles" in unicellular bacteria has been recognized only much more recently (reviewed in references 18 and 31). As part of this discovery process, the enzymes that synthesize and degrade c-di-GMP and the genes that encode them have been identified and characterized. Coupled with the exponential increase in bacterial genome sequencing, this has led to the realization that c-di-GMP signaling is ubiquitous in bacteria and that its global significance in bacterial physiology has still to be appreciated. c-di-GMP functions by acting as an effector ligand, directly controlling the activities of a disparate array of targets, including transcription factors, cellulose and alginate biosynthetic enzymes, riboswitches, proteins involved in proteolytic targeting, and complex cellular structures like the flagellar basal body (2, 4, 18, 19, 26, 28, 31, 37, 40, 44).

c-di-GMP signaling has been extensively studied in unicellular bacteria, such as *Escherichia coli*, *Salmonella*, and *Caulobacter crescentus* (18), showing that it regulates the decision to form either motile, planktonic cells or a sedentary biofilm ("swim or stick"). In *E. coli* and *Salmonella*, a high level of c-di-GMP reduces the expression and/or activity of flagella and stimulates the expression of adhesins, such as curli fimbriae, and of biofilm-associated exopolysaccharides, such as cellulose (39). In *C. crescentus*, cell division is asymmetric, producing a flagellated, motile swarmer cell and a sessile stalked cell that attaches to surfaces via a terminal structure called a holdfast. Only the stalked cell can divide; as a consequence, in order to proliferate, the motile swarmer cell must differentiate into a stalked cell, and this involves flagellum ejection, surface attachment through pili, and the synthesis of an adhesive holdfast and stalk at the same pole previously occupied by the flagellum. The role of c-di-GMP in this cell cycle progression has been investigated, showing that a polarly localized diguanylate cyclase called PleD is required for holdfast formation, flagellum ejection, and stalk formation (27, 30) and that a polarly localized c-di-GMP phosphodiesterase homolog called TipF is required for the assembly of flagella at the newly born pole after cell division (22). In addition, c-di-GMP controls virulence in animal and plant pathogens like *Vibrio cholerae* and *Xanthomonas campestris*, in which a drop in c-di-GMP levels caused by the activities of phosphodiesterases activates virulence factor production (34, 41).

Levels of c-di-GMP inside bacteria are regulated by two enzymes with opposing activities: diguanylate cyclases and phosphodiesterases (37). Diguanylate cyclases catalyze the formation of c-di-GMP from two molecules of GTP (30, 36, 37). In contrast, phosphodiesterases degrade c-di-GMP to form linear di-GMP (pGpG), which slowly and spontaneously hydrolyzes to give two molecules of GMP (5, 38). The characteristic activities of diguanylate cyclases are associated with a conserved domain carrying a Gly-Gly-Asp-Glu-Phe (GGDEF) motif, and similarly, the activities of phosphodiesterases are associated with a conserved domain carrying a Glu-Ala-Leu (EAL) motif. Recently, an additional domain, the [His-Asp]-[Gly-Tyr-Pro] (HD-GYP) domain has also been shown to be involved in the degradation of c-di-GMP to GMP in the plant pathogen *Xanthomonas campestris* (34). Proteins with GGDEF, EAL, and HD-GYP domains frequently also have sensing domains, such as PAS domains, involved in the perception of oxygen and redox potential (32), GAF domains, which bind cyclic mononucleotides (35), and response regulator receiver (REC) domains (20, 30), suggesting that the enzymatic synthesis or degradation of c-di-GMP is regulated in response to environmental cues (13). Further, a conserved motif called the I site (RXXD), located 5 amino acids (aa) upstream of the conserved A site (GGDEF), adds another layer of regulation to the cellular homeostasis of c-di-GMP, as revealed through the analysis of the *C. crescentus* diguanylate cyclases PleD and DgcA (6). Binding of c-di-GMP to the I site inhibits the diguanylate cyclase activity associated with the GGDEF domain in a noncompetitive manner, allowing feedback inhibition of c-di-GMP on its own synthesis (6, 37, 42).

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TABLE 1. Strains and plasmids used in this study

Strain or plasmid	Relevant genotype or comment[a]	Source or reference
Strains		
S. coelicolor		
M600	SCP1$^-$ SCP2$^-$	25
J3359	Δ*cdgB*::*apr* SCP1$^-$ SCP2$^-$	This work
E. coli		
ET12567/pUZ8002	ET12567 containing helper plasmid pUZ8002	29
Plasmids		
pMS82	Plasmid cloning vector for the conjugal transfer of DNA from *E. coli* to *Streptomyces* spp., integrated site specifically at the ΦBT1 attachment site (Hyg^r)	15
pIJ10257	Plasmid integrating at the ΦBT1 *attB* attachment site of *S. coelicolor* and containing the strong *ermEp** promoter (Hyg^r)	21
pDONR207	Gateway donor vector	Invitrogen
pHNGWA	Gateway T7 system-based cloning vector for protein overexpression	3
pIJ10346	pUC19 carrying *cdgB* driven by its native promoter	This work
pIJ10347	pMS82 carrying *cdgB* driven by its native promoter	This work
pIJ10350	pIJ10257 carrying *cdgB*	This work
pIJ10352	pIJ10257 carrying *cdgB*-FLAG	This work
pIJ10361	Like pIJ10352 but carrying the GGC^1-to-GCG mutation	This work
pIJ10362	Like pIJ10352 but carrying the GGC^2-to-GCG mutation	This work
pIJ10370	pHNGWA carrying *cdgB*	This work
pIJ10371	Like pIJ10370 but carrying the GGC^1-to-GCG mutation	This work
pIJ10372	Like pIJ10370 but carrying the GGC^2-to-GCG mutation	This work

[a] GGC^1 and GGC^2, the first and second GGC codons, respectively, of the conserved GGDEF motif.

Although c-di-GMP signaling has been extensively studied in unicellular bacteria that can alternate between motile and sessile lifestyles, c-di-GMP signaling has not been implicated in any aspect of the biology of the nonmotile, hyphal bacteria of the genus *Streptomyces*. When streptomycete spores germinate, one or two germ tubes emerge and grow by tip extension and branching to form a multicellular vegetative mycelium (12). Streptomycetes differentiate by forming specialized reproductive structures called aerial hyphae, which grow out of the aqueous environment of the vegetative mycelium into the air. Subsequently, each multigenomic aerial hypha undergoes a synchronous septation event, giving rise to ~50 to 100 unigenomic prespore compartments that ultimately develop into mature exospores (12). *bld* mutants are a class of developmental mutants that cannot erect aerial hyphae and therefore appear "bald," lacking the fuzzy morphology of the wild type (12). Streptomycetes are the most abundant source of clinically important antibiotics and other natural products used in human medicine, and the production of these molecules is temporally and genetically coordinated with the developmental program. Thus, in addition to causing loss of aerial-mycelium formation, mutations in many *bld* loci, including the *bldD* locus discussed in this report, pleiotropically block antibiotic production (8, 10, 12).

Here we show that CdgB (SCO4281), a direct target of the key developmental regulator BldD, acts as a diguanylate cyclase *in vitro*, that both overexpression and deletion of *cdgB* in *Streptomyces coelicolor* inhibit aerial-mycelium formation, and that overexpression also inhibits production of the antibiotic actinorhodin, implicating c-di-GMP in the regulation of developmental processes in *Streptomyces*.

MATERIALS AND METHODS

Strains, plasmids, and growth conditions. Bacterial strains and plasmids used in this study are described in Table 1. Unless described otherwise, media and culture conditions were as given previously, including recipes for soy flour mannitol (SFM), R2, and R5 media (25).

Conjugal transfer of plasmid and cosmid DNA from *E. coli* to *Streptomyces* spp. To bypass the methyl-specific restriction system of *S. coelicolor*, cosmids and plasmids were introduced into the *dam dcm hsdS E. coli* strain ET12567, carrying the nontransmissible, *oriT*-mobilizing "driver" plasmid pUZ8002 (29). Conjugations from *E. coli* to *Streptomyces* were carried out as described by Kieser et al. (25).

Bioinformatic analysis. Searches of the *S. coelicolor* genome for proteins containing GGDEF domains were carried out using the StrepDB database (http://strepdb.streptomyces.org.uk). Proteins were analyzed for domains associated with the conserved GGDEF domains using the SMART database (http://smart.embl-heidelberg.de/).

Construction of a *cdgB* null mutant derivative of *S. coelicolor* and complementation of a Δ*cdgB* mutant. A *cdgB* (SCO4281) null mutant allele in which the entire coding sequence was replaced with a cassette carrying the apramycin resistance gene (*apr*) and *oriT* of RK2 was constructed by the Redirect PCR-targeting method of Gust et al. (16). Cosmid StD95A, which carries *cdgB*, was introduced into *E. coli* BW25113/pIJ790, and *cdgB* was disrupted by electroporation of the cells with the *oriT-apr* cassette, which had been amplified using oligonucleotides containing *cdgB*-specific extensions (cdgBFW, 5′-CTT GAT TCA CTG CGA GGT CTC GGG GGG AGG GCG AGC ATG ATT CCG GGG ATC CGT CGA CC-3′; cdgBRV, 5′-GGT GAC TCG GAC CGC ATC AAC ACG CTG ACT TGG TGA TCA TGT AGG CTG GAG CTG CTT C-3′). Gene disruption was confirmed by restriction and PCR analyses of isolated cosmid DNA. The disrupted cosmid was introduced into the methylation-deficient *E. coli* strain ET1257/pUZ8002 and transferred by conjugation into *S. coelicolor* M600 (a plasmid-free derivative of the wild type), with selection for apramycin resistance. Null mutants generated by double crossing over were identified by their apramycin-resistant and kanamycin-sensitive phenotypes and were confirmed by PCR. A representative mutant was designated J3359.

To complement the *cdgB* mutant, a 3,647-bp AleI fragment was isolated from cosmid StD95A and cloned into dephosphorylated SmaI-cut pUC19. A 5.9-kb SalI fragment was isolated from this construct and self-ligated to generate pIJ10346. The *cdgB* gene carrying its native promoter was removed from pIJ10346 as a 3.3-kb KpnI-HindIII fragment and introduced into the integrative vector pMS82 to generate pIJ10347.

Overexpression and purification of CdgB from *E. coli*. The *cdgB* coding sequence was amplified by PCR using Phusion *Taq* polymerase (New England Biolabs) with a pair of primers (cdgBGWFW, 5′-GGG GAC AAG TTT GTA CAA AAA AGC AGG CTT AGA GAC CGA CTC GGA GCC CTA TGT CC-3′; cdgBGWRV, 5′-GGG GAC CAC TTT GTA CAA GAA AGC TGG

GTC CCG CAT CAA CAC GCT GAC TTG GTG-3′). The PCR product was initially cloned into the Gateway donor vector pDONR207 (Invitrogen) with BP Clonase (Invitrogen) and subsequently into pHNGWA (3) using LR Clonase (Invitrogen) to generate pIJ10370.

To create G472A and G473A alleles, *cdgB* in pDONR207 was used as a template for QuikChange site-directed mutagenesis (Invitrogen) using primers for G472A (5′-CGT GGC CCG GCT CGC GGG CGA CGA GTT CG-3′ and 5′-CGA ACT CGT CGC CCG CGA GCC GGG CCA CG- 3′) and G473A (5′-GGC CCG GCT CGG CGC GGA CGA GTT CGT G-3′ and 5′-CAC GAA CTC GTC CGC GCC GAG CCG GGC C-3′). The G472A and G473A alleles were moved into pHNGWA (3) using LR Clonase (Invitrogen) to generate pIJ10371 and pIJ10372, respectively.

pIJ10370, pIJ10371, and pIJ10372 were individually introduced into BL21(DE3)/pLysS, and *cdgB* expression was induced in exponentially growing cells at 30°C by the addition of 1 mM IPTG (isopropyl-β-D-thiogalactopyranoside). To purify CdgB, cells were collected 4 h after IPTG induction, resuspended in buffer A (10% glycerol, 50 mM Tris, 100 mM NaCl, 10 mM imidazole, pH 8), and subjected to sonication (4 cycles, 10-μm amplitude, 15 s on, 15 s off). The supernatant was collected by centrifugation at 13,000 × *g* at 4°C. CdgB was purified on a HisTrap column (GE Healthcare) and eluted with a gradient of imidazole (10 to 500 mM) in buffer B (10% glycerol, 50 mM Tris, pH 8, 100 mM NaCl). The purified protein was buffer exchanged into 10% glycerol, 50 mM Tris, pH 8, 100 mM NaCl using a Spin desalting column (Zeba) and stored at −80°C.

Overexpression of *cdgB* in *S. coelicolor*. The *cdgB* coding sequence was amplified by PCR using Phusion *Taq* polymerase (NEB) and a pair of primers incorporating NdeI and HindIII sites at their 5′ ends, respectively (cdgBOVERFW, 5′-CAT ATG GAG ACC GAC TCG GAG CC-3′, and cdgBOVERRV, 5′-AAG CTT ACG CTG ACT TGG TGA TCA TC-3′). To add a C-terminal FLAG tag, *cdgB* was amplified using the same forward primer (cdgBOVERFW) and the reverse primer cdgBOVERFLAGRV (5′-AAG CTT TCA CTT ATC GTC GTC ATC CTT GTA GTC TCC GGC GCG GCG GTG CTG TTTG-3′). The PCR products were cloned into dephosphorylated SmaI-cut pUC19 (Fermentas), sequenced, removed as NdeI-HindIII fragments, and subcloned downstream of the *ermEp** promoter (a strong, mutant variant of the wild-type *ermEp* promoter) in the ΦBT1 *attB* site-specific integrative vector pIJ10257 to generate pIJ10350 (*cdgB*) and pIJ10352 (*cdgB-FLAG*). To create G472A and G473A alleles, *cdgB* plus a C-terminal FLAG tag in pUC19 was used as a template for QuikChange site-directed mutagenesis (Invitrogen) using primers G472A (5′-CGT GGC CCG GCT CGC GGG CGA CGA GTT CG-3′ and 5′-CGA ACT CGT CGC CCG CGA GCC GGG CCA CG- 3′) and G473A (5′-GGC CCG GCT CGG CGC GGA CGA GTT CGT G-3′ and 5′-CAC GAA CTC GTC CGC GCC GAG CCG GGC C-3′). The G472A and G473A alleles were cloned into pIJ10257 to generate pIJ10361 and pIJ10362, respectively.

Preparation of crude cell extracts and CdgB immunoblot analysis. *S. coelicolor* was grown in tryptone soy broth (TSB)-yeast extract malt extract (YEME) (60:40, vol/vol) as described previously (25), and 5-ml samples were collected. Cells were harvested by centrifugation, washed with 5 ml ice-cold washing buffer (20 mM Tris, pH 8.0, 5 mM EDTA), and resuspended in 0.4 ml ice-cold sonication buffer (20 mM Tris, pH 8.0, 5 mM EDTA, 1× protease inhibitor [Roche]). Samples were sonicated immediately for 4 to 5 cycles, with 15 s on and 15 s off, at a 4.5-μm amplitude. Cell debris was removed by centrifugation at 9,000 × *g* for 15 min at 4°C, after which the protein concentration of the cleared extract was determined with Bradford reagent (Bio-Rad). Equal amounts (25 μg) of protein from each sample were loaded onto a 7.5% polyacrylamide gel and, after electrophoresis, transferred to a Hybond-C Extra nylon membrane (GE Healthcare) and probed with a 1:3,000 dilution of anti-FLAG antibodies (Sigma) that had been raised in rabbits. Using horseradish peroxidase-coupled secondary antibody, FLAG-tagged proteins were detected by enhanced-chemiluminescence (ECL) Western blotting detection according to the manufacturer's instruction (GE Healthcare).

***In vitro* diguanylate cyclase assays and detection of c-di-GMP using HPLC and liquid chromatography-mass spectrometry (LC-MS).** Diguanylate cyclase assays (600 μl) were essentially performed as described previously (30). *Caulobacter crescentus* PleD* (1, 30) was used as a positive control. In short, 20 μg CdgB or PleD* was incubated at 37°C for 2 h in 1× DGC buffer (10% glycerol, 50 mM Tris, pH 8, 10 mM $MgCl_2$, 100 mM NaCl) in the presence of 150 μM GTP (Promega). Separation of c-di-GMP was performed by strong-anion-exchange–high-performance liquid chromatography (SAX-HPLC). Protein was removed from reaction mixtures using Amicon filters (Millipore), and samples were injected onto a 4.6- by 250-mm, 10-μm-particle-size Partisil SAX column (HiChrom) run on an Agilent 1100 HPLC system fitted with a photodiode array detector. The mobile phases KH_2PO_4 (A) (7 mM adjusted to pH 4.0 with phosphoric acid) and KH_2PO_4 (B) (0.5 M containing Na_2SO_4, 0.5 M, adjusted to pH 5.4 with NaOH) were used at the following gradient: time zero, 0% B; 5 min, 15% B; 10 min, 19% B; 15 min, 50% B; 20 min, 70% B; 25 min, 75% B; and 30 min, 75% B, followed by 20 min of equilibration with 100% A.

To analyze reactions by LC-MS, samples (15 μl) were injected onto a Polar RP 150- by 2-mm column (Phenomenex) fitted onto a Surveyor HPLC system equipped with a Deca XP Plus ion trap mass spectrometer (Thermo) and separated with a gradient of 0.1% formic acid (A) and methanol (B) (0 min, 2% B; 2 min, 2% B; 15 min, 25% B; 19 min, 90% B; 22 min, 90% B; 22.5 min, 2% B; 34 min, 2% B) at a flow of 250 μl/min. MS data were collected by electrospray ionization in negative mode, with 50 units sheath gas, 5 units auxiliary gas, a 350°C capillary temperature, and a 5.2-kV spray voltage. Full MS and data-dependent MS2 and MS3 data were collected at 35% collision energy and an isolation width of 4.0 atomic mass units (amu). Nucleotide standards were obtained from Sigma Chemicals (GTP, GDP, GMP) and Biolog (c-di-GMP).

DNase I footprinting experiments. DNase I footprinting experiments were carried out essentially as described previously (8) and according to the description supplied with the Sure Track footprinting kit (GE Healthcare). A 167-bp probe spanning the promoter region of *cdgB* was generated by PCR using oligonucleotides 4281_F5 (5′-GAAACCCCACGCAATTGTTC-3′) and 4281_R5 (5′-GACCTCGCAGTGAATCAAGG-3′). Oligonucleotides were first end labeled using T4 polynucleotide kinase (New England BioLabs) and [γ-^{32}P]ATP (PerkinElmer) as described by the manufacturer. Prior to DNase I treatment, radioactive probes (approximately 110,000 cpm) were incubated with various amounts of histidine-tagged BldD, purified as described previously (9), at 30°C in a 40-μl volume containing 10 mM Tris-HCl (pH 7.8), 150 mM NaCl, 2 mM dithiothreitol, 1 μg of poly(dI · dC) (Roche), and 10% glycerol.

S1 nuclease mapping and G+A Maxam-Gilbert chemical sequencing. S1 nuclease mapping and G+A ladder sequencing were carried out essentially as described previously (25). The probe was generated by PCR using SCO4281S1FW (5′-ATCTCCGGGGCGTGGGCCGGAC-3′) and SCO4281S1RV (5′-GGCCTTG TTCATGTCGGCCATG-3′), which had been 5′-end labeled using T4 polynucleotide kinase (New England BioLabs) and [γ-^{32}P]ATP (PerkinElmer) as described by the manufacturer.

Congo red and crystal violet assays. Ten microliters of exponentially growing cells of *E. coli* BL21(DE3)/pLysS harboring pIJ10370 was dropped on an L agar plate containing 50 μg/ml Congo red (Sigma) in the presence or absence of 1 mM IPTG. Colony morphology was examined by visual inspection after 1 day at 37°C and then after 1 day at 30°C. Biofilm formation at the air-liquid interface was visualized by slow addition of 0.5% (wt/vol) crystal violet (Sigma) and incubation at room temperature for 30 min, followed by rinsing with water. For biofilm detection, cultures were grown for 4 h with shaking after induction with IPTG and then grown statically at 30°C for 10 days.

RESULTS

CdgB is one of eight GGDEF domain-containing proteins in *S. coelicolor*. Bioinformatic searches identified seven GGDEF domain-containing proteins encoded on the chromosome of *S. coelicolor* and one on the 356-kb linear plasmid SCP1. The domain architectures of these proteins were analyzed using the SMART database and are depicted in Fig. 1. Of the eight GGDEF domain proteins, four also contain EAL domains and two (CdgB and SCO5511) contain an RXXD motif (the I site) positioned 5 aa upstream of the GGDEF motif (the A site). In five of the eight proteins, the C-terminal GGDEF/EAL domains are preceded by GAF and/or PAS signaling domains. Two of the proteins (SCO5495 and SCO5511) have multiple predicted membrane-spanning helices.

Using chromatin immunoprecipitation with microarray (ChIP-chip) analysis, we recently showed that three of the GGDEF protein-encoding genes, *cdgA*, *cdgB*, and SCO5511, are direct targets of the developmental regulator BldD (Fig. 2A) (8). Overexpression of *cdgA* was found to influence both differentiation and antibiotic production (8). Here we investigated the functions of *cdgB*, further implicating c-di-GMP in the control of developmental processes in *Streptomyces*.

CdgB is a predicted 60.5-kDa cytoplasmic protein with a

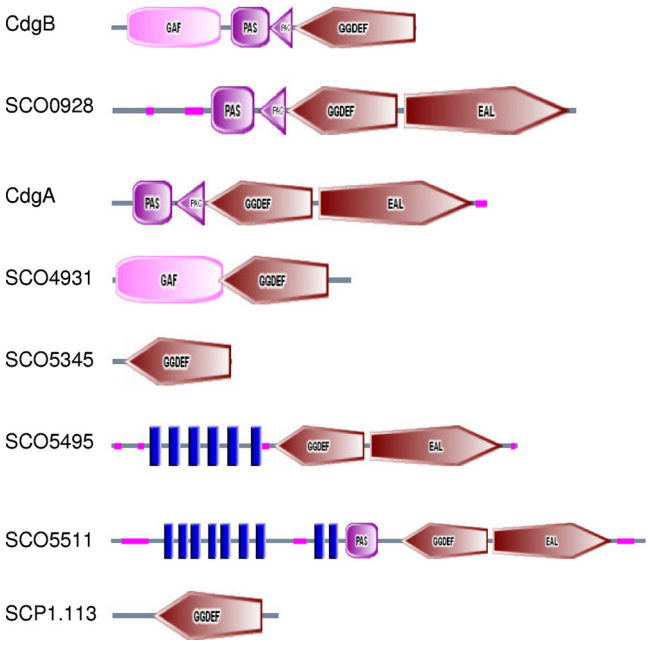

FIG. 1. Domain architectures of the eight proteins containing conserved GGDEF domains encoded in the genome of *S. coelicolor*, predicted using the SMART database. Predicted membrane-spanning helices are shown as blue vertical boxes, and segments of low compositional complexity are shown as pink horizontal boxes. In five of the eight proteins, the C-terminal GGDEF/EAL domains are preceded by GAF and/or PAS signaling domains.

C-terminal GGDEF domain preceded by PAS and GAF domains (Fig. 1), and the GGDEF motif is associated with an RXXD motif (an I site). CdgB is conserved in all the streptomycetes for which genome sequences have been published, e.g., *Streptomyces avermitilis* (85% identity), *Streptomyces griseus* (79% identity), *Streptomyces clavuligerus* (78% identity), and *Streptomyces scabies* (82% identity).

***cdgB* is a direct target of BldD.** To confirm and extend the ChIP-chip analysis (Fig. 2A), the BldD binding site in the *cdgB* promoter was mapped precisely using DNase I footprinting analysis (Fig. 2B). Incubation with purified histidine-tagged BldD protected a region centered approximately 110 bp upstream of the annotated ATG start codon of *cdgB*. Inspection of the BldD-protected region identified a sequence (TTGAC CCTGTGTACC) resembling the established 15-bp palindromic BldD consensus binding site, the BldD box [nTnACn C(A/T)GnGTnAn] (8). To determine the position of the BldD binding site relative to the promoter elements, the transcription start site of *cdgB* was mapped using S1 nuclease protection analysis (Fig. 2C). This revealed that the BldD binding site extends from −52 to −23 of the *cdgB* promoter, consistent with BldD functioning as a repressor of *cdgB* (Fig. 2D).

Disruption of *cdgB* delays morphological differentiation on R2 and R5 media but not on SFM. BldD directly regulates more than 20 genes known to be individually important for morphological differentiation and antibiotic production in *S. coelicolor* (8, 10, 24), suggesting that *cdgB* might also be involved in developmentally coordinated processes. To investigate this possibility, we constructed a *cdgB* null mutant in which the entire gene was replaced with a cassette conferring apramycin resistance. In liquid culture, the growth rate of the *cdgB* mutant was very similar to that of the wild type (data not shown). However, deletion of *cdgB* severely inhibited aerial-mycelium formation on R2 (Fig. 3A) and R5 media, but not on SFM medium. The developmental defect on R2 medium was still apparent after 6 days of incubation, especially at the single-colony level. This phenotype was fully complemented in *trans* when the wild-type *cdgB* gene, expressed from its native promoter, was introduced on the integrative, single-copy plasmid pMS82 (Fig. 3A).

Overproduction of *cdgB* inhibits aerial hyphal formation and antibiotic production on R5 and SFM media but not on R2. To determine if overexpression of *cdgB* influenced the morphological development of *S. coelicolor*, *cdgB* was placed under the control of the strong constitutive promoter *ermEp** in the integrative single-copy vector pIJ10257. Introduction of the *ermEp*-cdgB* construct severely inhibited aerial-mycelium formation on R5 (Fig. 3B) and SFM media but not on R2 medium. As with the *cdgB* mutant phenotype, the overexpression phenotype was particularly pronounced in single colonies. Although the strain retained the ability to produce the red-pigmented antibiotic undecylprodigiosin, it was severely affected in the production of the blue-pigmented antibiotic actinorhodin (Fig. 3B).

The GGDEF motif is required for the bald phenotype of the *cdgB*-overexpressing strain. The GGDEF motif is perfectly conserved in CdgB in all streptomycete orthologs (data not shown). To determine if the GGDEF signature is required for the inhibition of the aerial-mycelium formation phenotype of the overexpression strain, we individually replaced G472 and G473 with alanine, using site-directed mutagenesis. A FLAG tag was introduced at the C termini of the products of the *cdgB* alleles to be able to monitor the effect of mutagenesis on the stability of the resulting CdgB variants. When this FLAG tag was fused to a wild-type allele of *cdgB*, overexpression of *ermEp*-cdgB* still resulted in inhibition of aerial-mycelium formation and a severe decrease in actinorhodin production, indicating that the FLAG tag did not interfere with CdgB function (Fig. 4A). The G472A and G473A substitutions in the GGDEF motif completely reversed the developmental phenotype caused by overexpression of FLAG-tagged wild-type CdgB (Fig. 4A), suggesting that the GGDEF motif was essential for the CdgB overexpression phenotype. To determine if the stability of CdgB was affected by mutagenesis of the GGDEF motif, immunoblot analysis was carried out using anti-FLAG antibodies (Fig. 4B). The wild-type and mutant CdgB proteins were readily detected in crude cell extracts, confirming that the GGDEF motif is required for the inhibition of the aerial-mycelium formation phenotype caused by *cdgB* overexpression. Truncation of the entire GGDEF domain (removal of the last 128 amino acid residues of CdgB) also relieved the inhibition of aerial-mycelium formation associated with *cdgB* overexpression (data not shown).

CdgB synthesizes c-di-GMP *in vitro*. The presence of a GGDEF domain and a conserved I site (RXXD) suggested that CdgB might function as a diguanylate cyclase. To test this hypothesis, N- and C-terminally His-tagged versions of complete CdgB were overexpressed in *E. coli*. Despite our trying many induction conditions, the protein remained poorly soluble. However, fusion of a His_6-NusA tag to the N terminus of

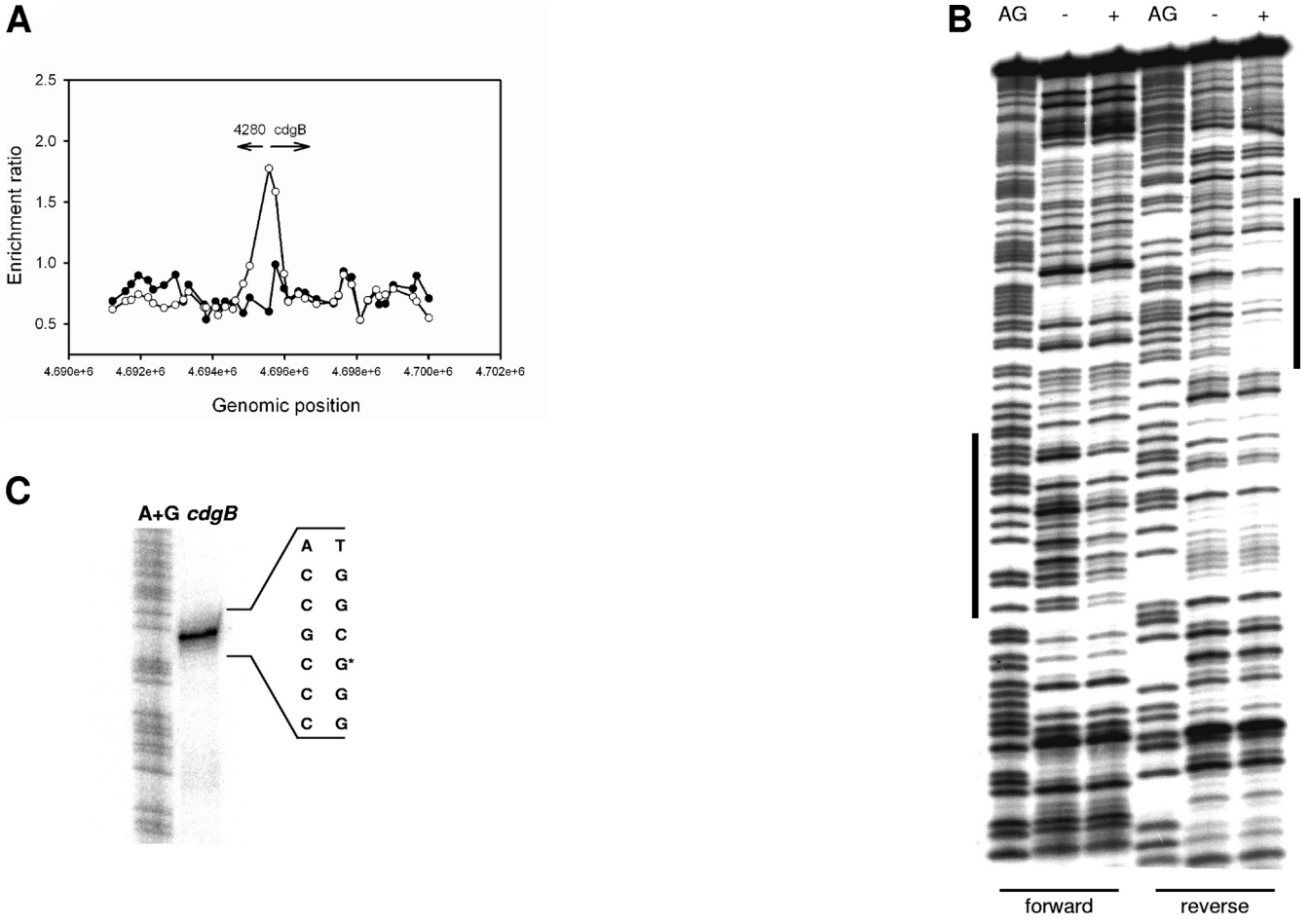

FIG. 2. (A) BldD ChIP-chip data for the 8-kb region spanning the *cdgB* locus in wild-type *S. coelicolor* (open circles) and the *S. coelicolor* Δ*bldD* mutant (filled circles). DNA obtained from immunoprecipitation of BldD was labeled with Cy3 and hybridized to DNA microarrays together with a total DNA control that was labeled with Cy5. Data are plotted as Cy3/Cy5 ratios (*y* axis) as a function of chromosome location (*x* axis). (B) DNase I footprinting analysis of BldD binding to the promoter region of *cdgB*. A 5′-end-labeled probe was incubated alone (−) or in the presence (+) of 5.5 μM BldD and subjected to DNase I footprinting analysis as described in Materials and Methods. Footprints are flanked by Maxam and Gilbert sequence ladders (AG). Protected regions are marked by bars. (C) High-resolution S1 nuclease mapping of the 5′ end of the *cdgB* transcript using a PCR-generated probe and RNA isolated from wild-type *S. coelicolor.* The most likely transcription start point is indicated by an asterisk. The G+A Maxam-Gilbert chemical sequencing ladder was generated with the same probe used for S1 nuclease mapping. (D) Summary of the DNase I footprinting and S1 nuclease mapping results presented in panels B and C, respectively. The DNase I-protected regions are bracketed, the bioinformatically predicted BldD binding sequence is depicted in bold, the *cdgB* transcription start point is indicated by the arrow, and the putative −35 and −10 sequences are underlined.

complete CdgB markedly improved solubility (data not shown), as has been found for other, unrelated proteins (3). Soluble His_6-NusA-CdgB was purified by nickel affinity chromatography (data not shown) and assayed *in vitro* for DGC activity in the presence of 150 μM GTP (Fig. 5). As negative controls, we purified two mutant versions of His_6-NusA-CdgB carrying either the G472A or the G473A substitution in the GGDEF motif of CdgB. As a positive control, we used purified PleD*, a mutant variant of the polar development regulator PleD, the well-studied diguanylate cyclase from *C. crescentus* (1, 30). Wild-type PleD requires activation by phosphorylation on an N-terminal REC domain, but PleD* has constitutive diguanylate cyclase activity in the absence of phosphorylation (1, 30). Reaction products were analyzed by HPLC, and peaks with retention times identical to that of a c-di-GMP standard were observed for both PleD* and CdgB (GGDEF) (Fig. 5A and B). This peak was absent in the reactions using either CdgB (AGDEF) or CdgB (GADEF) (Fig. 5C and D). GDP appeared as a spontaneous degradation product of GTP. The formation of c-di-GMP in the CdgB (GGDEF) reaction mixture was confirmed by LC-MS. The expected $[M-H]^-$ ion of c-di-GMP (*m*/*z* 689) was clearly detected in the CdgB reaction

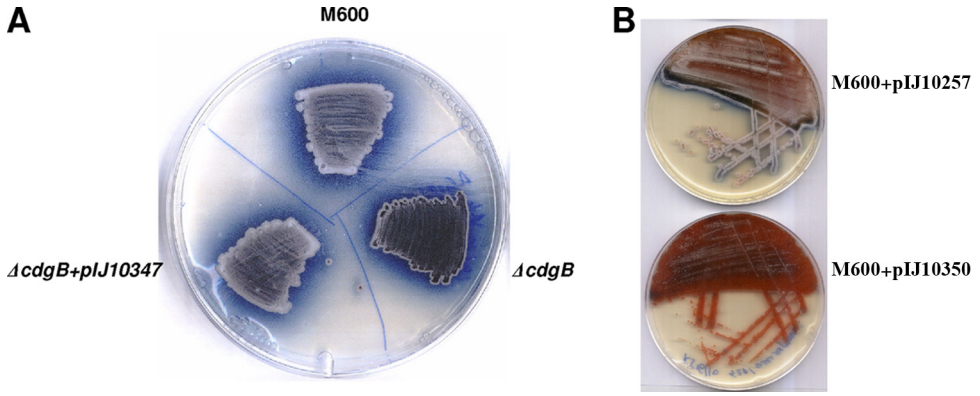

FIG. 3. Phenotypes of the constructed *cdgB* null mutant and the *cdgB*-overexpressing strain. (A) Wild-type *S. coelicolor* M600, its congenic *cdgB* mutant, and the complemented mutant grown on R2 medium for 3 days. Disruption of *cdgB* resulted in a severe delay in aerial-mycelium formation. (B) Wild-type *S. coelicolor* M600 carrying either pIJ10350 (*ermEp*-cdgB*) or the empty parent vector pIJ10257 and grown on R5 medium for 3 days. Overexpression of *cdgB* inhibited aerial-mycelium formation and severely reduced actinorhodin production.

(Fig. 5E), and its MS2 fragmentation pattern matched that of the c-di-GMP standard and of the control reaction with PleD* (data not shown). These assays demonstrate that CdgB is a diguanylate cyclase that can convert GTP to c-di-GMP and that mutagenesis of CdgB (G472A, G473A) blocked the enzyme activity of wild-type CdgB.

CdgB overexpression in *E. coli* causes changes in colony surface structure. In bacteria such as *Escherichia* and *Salmonella*, changes in intracellular c-di-GMP levels have profound effects on colony morphology and the production of extracellular matrix (23, 39, 43). These effects arise because c-di-GMP activates the production of two of the major components of the extracellular matrix produced by *Salmonella* and *Escherichia*, cellulose and the curli fimbriae (39, 46). Based on these observations, we expressed *cdgB* in *E. coli* BL21 from an IPTG-inducible promoter and monitored changes in colony phenotype in the presence of Congo red, which stains polysaccharides and proteinaceous attachment factors in the extracellular matrix. In the presence of 1 mM IPTG, the *E. coli* colonies showed increased binding of Congo red relative to that of the uninduced strain (Fig. 6A), indicating enhanced production of exopolysaccharide.

We also followed the effect of *cdgB* induction on changes in biofilm formation by evaluating the intensity of crystal violet staining in *E. coli* cells adhering to the glass surface at the air-liquid interface in static cultures (Fig. 6B). Cultures grown in the presence of increasing amounts of IPTG displayed enhanced crystal violet staining compared to the control sample. These observations are consistent with the hypothesis that expression of CdgB in *E. coli* causes enhanced production of c-di-GMP, which in turn leads to increased production of extracellular matrix.

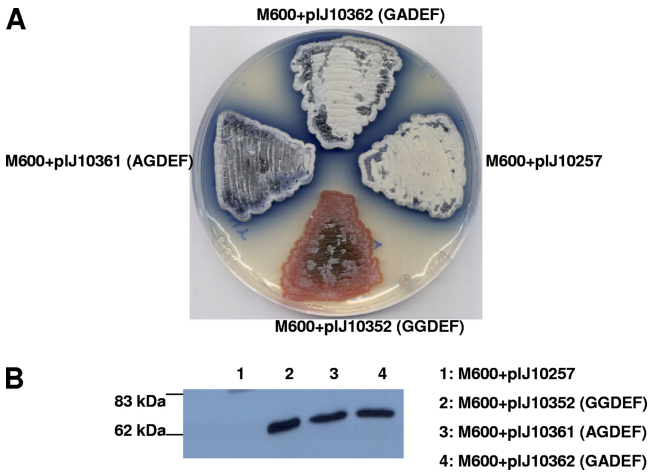

FIG. 4. (A) Mutagenesis of the conserved GGDEF motif of CdgB reverses the developmental phenotype of the CdgB-overexpressing strain and restores wild-type levels of actinorhodin production. Strains were incubated on R5 medium for 3 days. (B) Immunoblot analysis using anti-FLAG antibodies, showing CdgB protein levels in crude cell extracts of the strains from panel A. The positions of size markers are indicated to the left.

DISCUSSION

We recently showed that BldD, a key developmental regulator in *Streptomyces*, directly regulates three genes encoding GGDEF domain proteins, SCO5511, *cdgA*, and *cdgB* (Fig. 1) (8). Here we present biochemical and physiological evidence that CdgB is a diguanylate cyclase and show that both deletion and overexpression of *cdgB* inhibit aerial-mycelium formation in a growth medium-dependent manner and that overexpression also inhibits production of the antibiotic actinorhodin, unambiguously establishing a role for c-di-GMP in *Streptomyces* biology. In c-di-GMP biology, gene disruption phenotypes are generally held to be more significant than overexpression phenotypes because overexpression of guanylate cyclases can have indirect effects; an excess c-di-GMP can "spill over" from the local system that it normally controls and ectopically activate other unrelated c-di-GMP-regulated systems (14, 18). Overexpression of *cdgA* also inhibits aerial-mycelium formation and strongly inhibits actinorhodin production (8), but deletion of *cdgA* has no obvious phenotypic consequences (N. T. Tran, unpublished data).

In CdgB, the C-terminal GGDEF domain is preceded by PAS and GAF domains, implying that CdgB might depend on the binding of specific ligands or signal transduction for its output domain to become activated. Further, CdgB possesses a

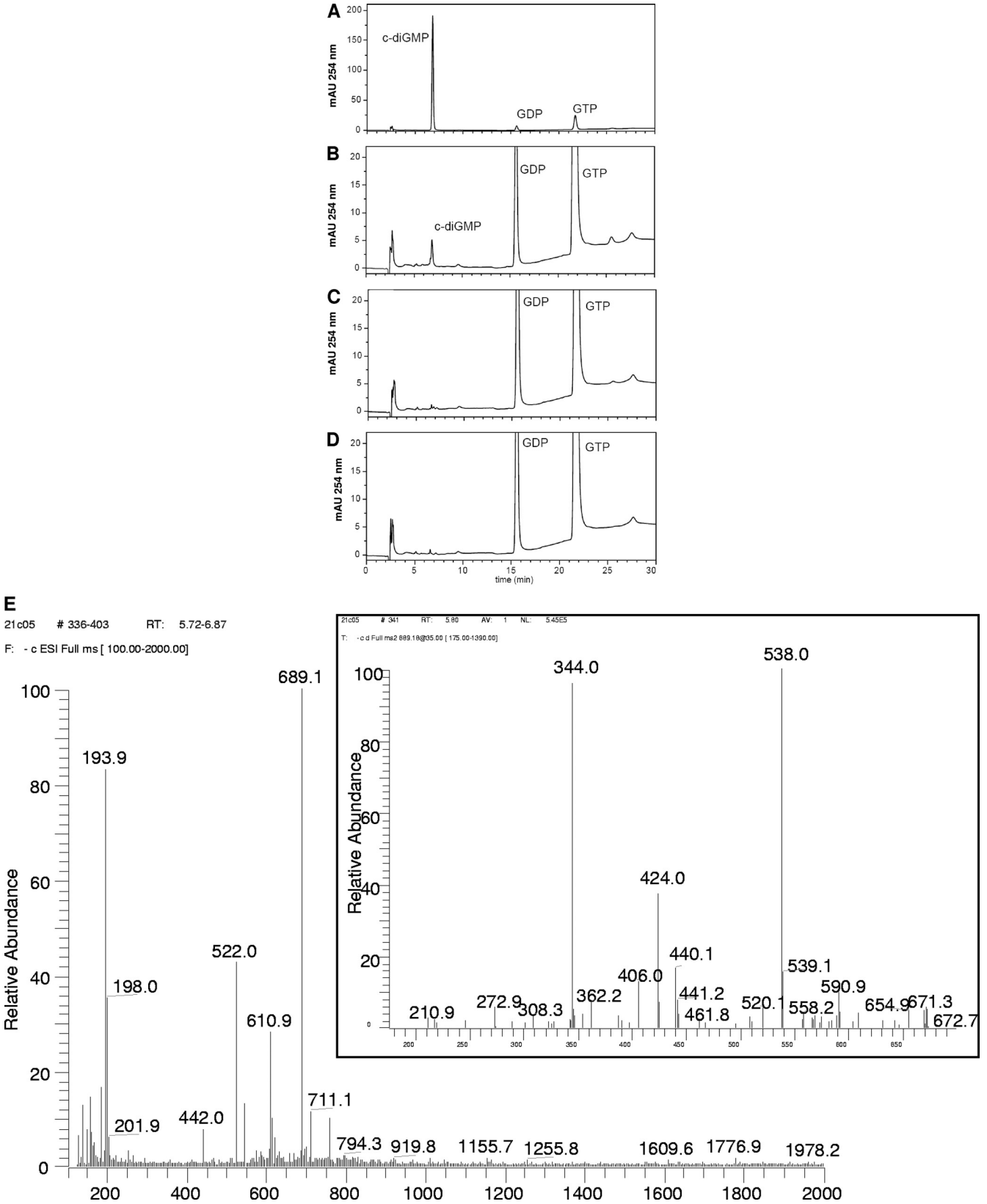

FIG. 5. HPLC detection of c-di-GMP production in diguanylate cyclase reaction mixtures containing PleD* (A), His_6-NusA-CdgB (GGDEF) (B), His_6-NusA-CdgB (AGDEF) (C), or His_6-NusA-CdgB (GADEF) (D). (E) LC-MS analysis of the c-di-GMP synthesized by CdgB. The expected $[M-H]^-$ ion of c-di-GMP (*m*/*z* 689) was clearly detected in the CdgB reaction, and its MS2 fragmentation pattern (inset) contained the expected fragments (*m*/*z* 538, 440, 424, and 344).

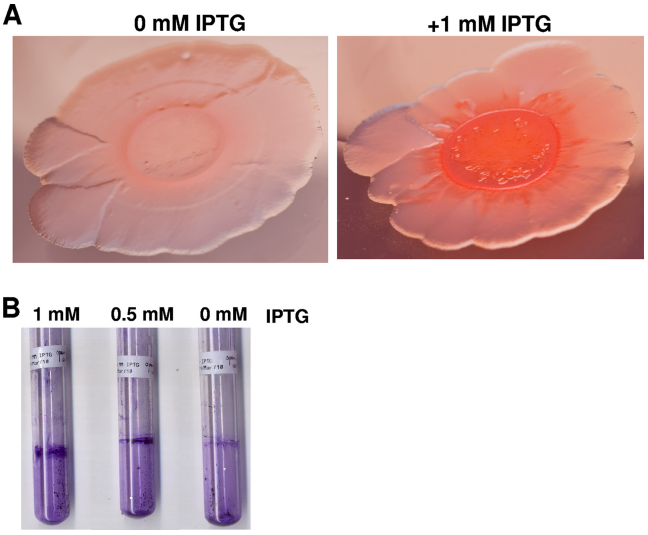

FIG. 6. Effects of heterologous expression of *cdgB* on *E. coli*. (A) Congo red binding and colony morphology. Colonies were grown on L agar in the presence of 50 μg/ml Congo red, with or without IPTG to induce *cdgB* expression. The plates were incubated for 1 day at 37°C, followed by 1 day at 30°C. (B) Biofilm formation. Static liquid cultures were grown in LB broth at 30°C for 10 days in the presence of different concentrations of IPTG, and adhesion of cell aggregates at the air-liquid interface was detected using crystal violet staining (0.5%, wt/vol).

conserved I site (RXXD) located 5 aa upstream of the GGDEF motif, implying, by analogy with PleD and DgcA (6), that c-di-GMP will interact with the I site, leading to negative regulation of its own production by feedback inhibition. It is somewhat puzzling that both deletion and overexpression of *cdgB* lead to similar phenotypes—a pronounced delay in aerial-mycelium formation—even given that the two effects are most pronounced on different media. If CdgB contained a phosphodiesterase (EAL) domain and a cyclase (GGDEF) domain, then overexpression might lead to a change in dominance between the two activities, but CdgB has no EAL domain. Nevertheless, the mutagenesis of the GGDEF motif implies that the overexpression phenotype is associated with c-di-GMP production, and the phenotype of the deletion mutant was fully complemented in single copy in *trans* by the wild-type *cdgB* gene.

In *E. coli* and *Salmonella*, c-di-GMP enhances the production of cellulose in the extracellular matrix (14, 39, 46). No such connection has been established in *Streptomyces*. However, *S. coelicolor* synthesizes an extracellular matrix containing cellulose, and disruption of *cslA*, encoding the putative cellulose synthase (CslA), causes a severe delay in the formation of aerial hyphae (7, 45), like deletion or overexpression of *cdgB*. Further, *cdgB* lies immediately upstream of SCO4282, a gene encoding a membrane protein with a predicted extracellular cellulose binding domain, although the significance of this juxtaposition is also not established. In *S. coelicolor*, CslA localizes to the tips of growing hyphae and interacts with DivIVA, an essential cytoskeletal protein that directs apical growth in *Streptomyces* (11, 17, 45). Xu et al. (45) proposed that cellulose fibers are secreted at the hyphal apex, perhaps adding rigidity and support to the vulnerable tips of growing hyphae. Recently, de Jong et al. (7) established an important role for cellulose in *Streptomyces*. They showed that *Streptomyces* hyphae attach to surfaces via amyloid fimbriae composed principally of hydrophobic proteins called chaplins and provided strong evidence that these fimbriae are anchored to the surfaces of the hyphae by cellulose fibers.

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